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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,230,991**, on May 11, 1998, by **MCGILL UNIVERSITY**, assignee of Moshe Szyf, Sanjoy Bhattacharya and Shyam Ramchandani, for "DNA Demethylase, Therapeutic and Diagnostic Uses Thereof".

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ABSTRACT OF THE INVENTION

The present invention relates to a DNA demethylase enzyme having about 50 to about 60 KDa, and wherein said DNA demethylase enzyme is overexpressed in cancer cells and not in normal cells. The present invention also relates to the therapeutic and diagnostic uses of the DNA demethylase.

DNA DEMETHYLASE, THERAPEUTIC AND DIAGNOSTIC USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a novel enzyme, DNA demethylase, therapeutic and diagnostic uses thereof.

(b) Description of Prior Art

Previous results have demonstrated that mammalian cells bear a *bona fide* demethylase (dMTase). Whereas a large body of data has shown that demethylation of DNA occurs *in vivo* and plays an important role in cellular differentiation and vertebrate development (Paroush, Z.et al. (1990) *Cell* **63**:1229-1237), the identity of this enzyme has remained a mystery.

The common accepted consensus among the leaders in the field of DNA biochemistry has been that removal of a methyl group from 5-methyl-cytosine which must involve a cleavage of a C-C bond is impossible because of the high energy required for the process. Therefore, alternative enzymatic process that can replace methylated cytosine with cytosine were sought after. It was suggested that a glycosylase which cleaves the bond between the deoxyribose and the 5-methylcytosine is involved in demethylation (Jost, J. P. et al. (1995) *J. Biol. Chem.* **270**:9734-9739).

Others have suggested that demethylation occurs by an RNA-catalyzed process and involves replacement of the dinucleotide methyl-CpG with CpG (Weiss, A. et al. (1996) *Cell* **87**:709-718).

It would be highly desirable to be provided with a *bona fide* DNA demethylase (DNA dMTase).

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a *bona fide* DNA demethylase (DNA dMTase).

In accordance with the present invention there is provided a DNA demethylase enzyme having about 50 to about 60 KDa, and wherein the DNA demethylase enzyme is overexpressed in cancer cells and not in normal cells.

In accordance with the present invention there is provided a cDNA encoding human demethylase, which comprises a sequence set forth in Fig. 4.

In accordance with the present invention there is provided a cDNA homologous to the human cDNA, wherein the cDNA encoding mouse demethylase.

In accordance with the present invention there is provided the use of the expression of demethylase cDNAs to alter DNA methylation patterns of DNA *in vitro* in cells or *in vivo* in animals and in plants.

The demethylase cDNA expression may be under the direction of mammalian promoters, such as CMV.

The demethylase cDNA expression may be under plant specific promoters to alter methylation in plants and to allow for altering states of development of plants and expression of foreign genes in plants.

The demethylase cDNA expression may be in the antisense orientation to inhibit demethylase in cancer cells for therapeutic processes.

The expression of demethylase cDNA in mammalian cells may be to alter their differentiation state and to generate stem cells for therapeutics, cells for animal cloning and to improve expression of foreign genes.

In accordance with the present invention there is provided the use of the expression of demethylase cDNAs in bacterial or insect cells for production of large amounts of demethylase.

In accordance with the present invention there is provided the use of the expression of demethylase cDNAs for the production of protein in vertebrate, insect or bacterial cells, such as antibodies against demethylase.

In accordance with the present invention there is provided the use of the sequence of demethylase cDNA as a template to design antisense oligonucleotides and ribozymes.

In accordance with the present invention there is provided the use of the predicted peptide sequence of demethylase cDNA to produce polyclonal or monoclonal antibodies against demethylase.

In accordance with the present invention there is provided the use of expression of cDNAs in two hybrid systems in yeast to identify proteins interacting with demethylase for diagnostic and therapeutic purposes.

In accordance with the present invention there is provided the use of expression of cDNAs in bacterial, vertebrate or insect cells to produce large amounts of demethylase for high throughput screening of demethylase inhibitors for therapeutics and biotechnology.

In accordance with the present invention there is provided a volatile assay for high throughput screening of demethylase inhibitors as therapeutics and anticancer agents which comprises the steps of:

a) using transcribed and translated demethylase cDNAs *in vitro* to convert methyl-cytosine present in methylated DNA samples to cytosine present in DNA and volatilise methyl group;

b) determining the absence or minute amount of volatilise methyl group as an indication of an active demethylase inhibitor.

In accordance with the present invention there is provided a volatile assay for the diagnostics of cancer in a patient sample which comprises the steps of:

a) using transcribed and translated demethylase cDNAs *in vitro* to convert methyl-cytosine present in methylated DNA patient sample to cytosine present in DNA and volatilise methyl group;

b) determining the presence or minute amount of volatilise methyl group as an indication of cancer in the patient sample.

In accordance with the present invention there is provided the use of an antagonist or inhibitor of DNA demethylase for the manufacture of a medicament for cancer treatment, for restoring an aberrant methylation pattern in a patient DNA, or for changing a methylation pattern in a patient DNA.

Such an antagonist is a double stranded oligonucleotide that inhibits demethylase at a Ki of 50nM, such as $\begin{pmatrix} C^mGC^mGC^mGC^mG \\ G^mCG^mCG^mCG^mC \end{pmatrix}_n$.

The inhibitor include, without limitation an anti-DNA demethylase antibody or an antisense of DNA demethylase.

The change of the methylation pattern may activate a silent gene. Such an activation of a silent gene permits the correction of genetic defect such as found for β-thalassemia or sickle cell anemia.

The DNA demethylase of the present invention may be used to remove methyl groups on DNA *in vitro*.

The DNA demethylase of the present invention or its cDNA may be used, for changing the state of differentiation of a cell to allow gene therapy, stem cell selection or cell cloning.

The DNA demethylase of the present invention or its cDNA may be used, for inhibiting methylation in cancer cells using vector mediated gene therapy.

In accordance with the present invention there is provided an assay for the diagnostic of cancer in a patient, which comprises determining the level of expression of DNA demethylase of the present invention in a sample from the patient, wherein overexpression of the DNA demethylase is indicative of cancer cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the volatilization of methanol from methylated DNA by DNA dMTase;

Fig. 2 illustrates the volatile and CpG assay of Fraction 63 purified using gel electrophoresis;

Fig. 3 illustrates the alignment between the MDB domain of MECP-1 and demethylase;

Fig. 4 illustrates the sequence of human demethylase cDNA and its corresponding translation (amino acid sequence);

Fig. 5 illustrates the Northern blot analysis of DNA dMTase expression from different human tissue;

Fig. 6 illustrates the *in vitro* translated demethylase;

Fig. 7 illustrates the putative demethylase cDNA inserted in the antisense orientation into the pcDNA3.1/His Xpress vector;

Fig. 8 illustrates the inhibition of dMTase *in vitro* by a specific inhibitor;

Fig. 9 illustrates the removal and volatilization of $[^3H]CH_3$ from methyl CpG DNA catalyzed by *in vitro* translated demethylase;

Fig. 10 illustrates the demethylation of methylated CpG DNA by *in vitro* translated demethylase and DEAE purified demethylase; and

Fig. 11 illustrates the demethylation of plasmid pBluescript SK II by *in vitro* translated demethylase.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, it is shown and demonstrated that demethylation occurs by removal of a methyl group from methylated cytosine in DNA, that a hydrogen from water replaces the methyl group at the 5' position, that the resulting methyl group reacts with the remaining hydroxyl from water to generate methanol which volatilises (Fig. 1). Thus, *bona fide* demethylation of DNA involves the following reaction:

$$CH_3\text{-cytosine-(DNA)} + H\text{-}OH \xrightarrow{\text{demethylase}} H\text{-cytosine} + CH_3\text{-}OH$$

The cDNA cloned in accordance with the present invention is the demethylase since it can convert methyl-cytosines in DNA to cytosines and volatilise the methyl groups on DNA when transcribed and translated *in vitro*. This is a novel cDNA encoding a biochemical activity that has been not described before.

Purification of demethylase from cells and tissues

Nuclear extracts were prepared from A549 cultures at near confluence as previously described (Szyf, M. et al. (1991) *J. Biol. Chem.* **266**:10027-10030; Szyf, M. et al. (1995) *J. Biol. Chem.* **270**:12690-12696). The cells were trypsinized, collected and washed with phosphate-buffered saline and suspended in buffer A (10 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, 5mM KCl, 0.5% NP-40) at the concentration of 108 cells per ml for 10 min. at 4°C. Nuclei were collected by centrifugation of the suspension at 1000 g for 10 minutes. The nuclear pellet was resuspended in buffer A (400 µl) and collected as

above. A nuclear extract was prepared from the pelleted nuclei by suspending them in buffer B (20 mM Tris, pH 8.0, 25% glycerol, 0.2 mM EDTA and 0.4 mM NaCl) at the concentration of 3.3×10^8 per ml and incubating the suspension for 15 min. at 4°C. The nuclear extract was separated from the nuclear pellet by centrifugation at 10,000g for 30 minutes.

A freshly prepared nuclear extract (1 ml, 1.1 mg) was applied onto a DEAE-Sephadex™ column (Pharmacia) (1.0 x 2cm) that was preequilibrated with buffer L (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) at a flow rate of 1 ml/min. The column was then washed with 15 ml of the starting buffer (buffer L) and proteins were eluted with 5 ml of a linear gradient of NaCl (0.2-5.0 M). 0.8 ml fractions were collected and assayed for demethylase activity. Demethylase eluted between 4.9-5.0 M NaCl. The DEAE active demethylase fraction was size fractionated using the electrophoretic vertical gel purification apparatus (Tyler research Instruments). Active fraction was applied onto a 4% nondenatured acrylamide column which was laid on top of a 12% acrylamide gel in the lower tube of the apparatus. Electrophoresis was performed in the presence of nondenaturing standard electrophoresis buffer for three hours at 300 volt. The electrophoretically separated fractions were flushed out of the tube at a flow rate of 0.1 ml/second for 20 sec at three minutes intervals. The protein samples were analyzed for dMTase activity.

Assay of demethylase activity

To directly assay demethylase activity *in vitro* two independent methods were applied.

(A) To assay the conversion of methyl-dCMP (mdCMP) to dCMP we used a previously described method (Szyf, M. et al. (1995) *J. Biol. Chem.* **270**:12690-12696). Briefly, $\alpha^{32}P$ labeled, fully methylated poly[mdC^{32}PdG]n substrate was prepared as follows. One hundred ng of a double-stranded fully methylated (mdCpdG) oligomer (Pharmacia) were denatured by boiling, which was followed by partial annealing at room temperature. The complementary strand was extended with Klenow fragment (Boehringer Mannheim) using methyl-5-dCTP (mdCTP) (Boehringer Mannheim) and [α-^{32}P] GTP (100 µCi, 3000 Ci/mmol), and the unincorporated nucleotides were removed by chromatography through a NAP-5 column (Pharmacia) the NAP-5 chromatography was repeated to exclude minor contamination with unincorporated nucleotides. As a control a nonmethylated poly[dC^{32}pdG]n substrate was similarly prepared except that a nonmethylated dCpdG oligomer served as a template and dCTP was used in the extension reaction. The column fractions (30 µl), described above were incubated with 1 ng of poly[mdC^{32}pdG]n substrate for 1 hour at 37°C in a buffer L containing 25% glycerol (v/v) and 5 mM EDTA. The reacted DNA as well as a nonmethylated poly[dC^{32}pdG]n and methylated [mdC^{32}pdG]n nonreacted controls were purified by phenol/chloroform extraction and subjected to micrococcal nuclease digestion (100 µg at 10 µl) and calf spleen phosphodiesterase (2µg) (Boehringer) (Pharmacia) to 3' mononucleotides for 15 hours at 37°C. The digestion products were loaded onto a thin layer chromatography plate (TLC) (Kodak, 13255 Cellulose), developed in a medium containing, 132 ml Isobutyric acid:40 ml water:

4 ml ammonia solution, autoradiographed and the intensity of the different spots was determined using a phosphorimager (Fuji, BAS 2000). ^{32}P labeled substrates and tritium labeled substrates were phosphoimaged using BAS™ 2000 plate and BAS-TR2040™ phosphorimager plate respectively.

(B) The second method determined removal of methylated residues from methylated DNA by measuring volatilization of methyl groups from the DNA and their capture by liquid scintillation fluid in the outer chamber. 100 ng of poly [dCdG]n double stranded DNA was methylated using SssI methylase (New England Biolabs) and an excess of [^{3}H-methyl AdoMet (80 Ci/mmol; New England Nuclear)]. The tritiated methyl group containing DNA was purified from labeled AdoMet using NAP-5 column chromatography. All column purified fractions of demethylase were assayed using the tritiated substrate. In a typical assay, 1 ng of DNA was incubated (at a specific activity of 4 $x10^{6}$ dpm/mg) with 30 µl of demethylase fractions in buffer L in a 0.5 ml eppendorf tube. The tube was uncapped and placed floating in a sealed 10 ml scintillation vial containing 5 ml of scintillation liquid and incubated for an overnight at 37°C. In the absence of demethylase activity the CH_3 groups are covalently attached to DNA, since there is no contact between the DNA and the scintillation fluid, no counts are registered in the scintillation counter even after long incubation. In the presence of demethylase the [^{3}H]-CH_3 groups are volatilised as methanol and are transferred to volatile phase and mix in the liquid scintillation cocktail emitting radioactive counts. A spectrum analysis is performed to ascertain that the

spectrum of the disintegration events corresponds to the expected spectrum of [^{3}H]. No counts with a spectrum corresponding to [^{3}H] are observed when the [^{3}H]-CH_3-DNA is incubated in the absence of demethylase. We have not observed false positives in more than two hundred assays performed in our laboratory.

Measurements of methanol production catalyzed by demethylase by gas chromatography

Gas chromatography was performed with a Varian™ model 3400 GC equipped with a 30m Stabilwax™ column (0.053 cm i.d.: Restek Corporation). Nitrogen™ was used as carrier gas at a flow rate of 32 ml/min, the injector and detector chambers were at 200 and 300°C respectively. The column was maintained at 40°C for 5 minutes after sample injection.

The demethylase reaction was performed in eppendorf tubes kept within sealed scintillation vials with 300 µl of water as aqueous phase (in radioactive trapping experiments this was replaced by 300 µl of methanol). The demethylase reaction was initiated in buffer L (10 mM $MgCl_2$, 10 mM Tris-HCl pH 8.0) with 500 ng of tritiated SK plasmid (6000 dpm/µl) and 100 µl of demethylase at 37°C. After overnight incubation at 37°C, the aqueous phase surrounding the eppendorf tube was transferred to a fresh eppendorf tube, 2 µl of this mixture was injected in the gas chromatography using a gas tight syringe (Hamilton, Reno, Nevada).

Coupled *in vitro* transcription translation

The mRNAs encoded by the pcDNA 3.1/His Xpress demethylase constructs described above were transcribed and translated by coupled transcription-translation using Promega™ TNT reticulocyte lysate kit (according to manufacturer's protocol), 2 µg of each construct and 40µCi of [^{35}-S]methionine (1,000Ci/mmol, Amersham) in a 50µl reaction volume. To purify non labeled *in vitro* translated demethylase, coupled *in vitro* transcription and translation was performed as above but in the presence of cold methionine. The translation products were bound to a Probond™ nickel column (Invitrogen) and demethylase was eluted according to the manufacturer's protocol with increasing concentrations of imidazole. Demethylase is eluted at 350-500mM imidazole. The imidazole eluted demethylase was dialyzed and concentrated by lyophilization.

Cloning and construction of demethylase expression vectors

PCR amplification of the MBD domain of the putative demethylase candidate cDNA

One µg of total RNA prepared from the human small lung carcinoma cell line A549 was reverse transcribed using Superscript reverse transcriptase and random primers (Boehringer) in a 25 µl reaction volume according to conditions recommended by the manufacturer (GIBCO-BRL). Five µl of reverse transcribed cDNA were subjected to an amplification reaction with Taq polymerase (Promega, 1 unit) using the following set of primers: sense 5'CTGGCAAGAGCGATGTC 3', antisense 5'AGTCTGGTTTACCCTTATTTTG 3'.

Amplifications conditions were: step 1. 95°C 1 min.; step 2: 94°C 0.5 min; step 3: 45°C 0.5 min.; step 4: 72°C 1.5 min; steps 2-4 were repeated 30 times. $MgCl_2$ was adjusted to 1 mM according to conditions recommended by the manufacturer. The PCR products were cloned in pCR2.1 vector (InVitrogen) and the sequence of the cDNAs was verified by dideoxy-chain termination method using a T7 DNA sequencing kit (Pharmacia). The amplified fragment was excised from the plasmid with EcoRI, labeled with a Boehringer random prime labeling kit according to manufacturer's protocol and alpha ^{32}P-dCTP. The labeled probe was used to screen a HeLa cell cDNA library in λTriplEx phage (Clontech) according to standard procedures. Positive clones were identified and further purified by serial dilutions for 4 rounds. The insert in the pTriplEx plasmid was excised from the phage according to manufacturer's protocols and the identity of the insert was verified by sequencing. The insert was excised by NotI restriction and subcloned into either the inducible expression vector: Retro tet on (Clontech) in the sense and antisense orientation or the pcDNA3.1/His Xpress vector in all three frames and in the antisense orientation.

Transfection and expression of demethylase in vertebrate cells

Ten µg of either Retro tet on demethylase or pcDNA 3.1/His Xpress demethylase are mixed with 8 µl of transfection lypophilic reagent Pfx-2 (Invitrogen) and placed upon 100,000 mouse (3T3 Balb/c, human (A549) or monkey cells (CV-1) according to manufacturer's protocol in OPTIMEM medium for 4 hours. Cells are harvested after 48 hours and demethylation and

demethylase activity is determined by measuring total genomic DNA methylation using standard techniques or a cotransfected *in vitro* methylated plasmid using a HpaII /MspI restriction enzyme analysis. Cellular transformation is measured by a soft agar assay.

Demethylation of pBluescript SK(+) Plasmid

About 4 µg plasmid pBluescript SK (Stratagene) was subjected to methylation using SssI methylase. The methylated plasmid (4 ng) was incubated for different time points as indicated with 30 µl of DNA dMTase Fraction 4 of DEAE-Sephacel™ column under standard conditions, extracted with phenol: chloroform and precipitated with ethanol. About 1 ng of the plasmid were subjected to digestion with 10 units each of either of the restriction endonuclease EcoRII (GIBCO-BRL), DpnI, or HpaII (New England Biolabs) before and after methylation as well as after DNA dMTase treatment in a reaction volume of 10 µl for 2 hour at 37°C. Following restriction digestion the plasmids were extracted with phenol:chloroform, ethanol precipitated and resuspended in 10 µl. The plasmids were electrophoresed on a 0.8% (w/w) Agarose gel, transferred onto a Hybond™ Nylon membrane and hybridized with pBluescript SK(+) plasmid which was ^{32}P labeled by random-priming (Boehringer Mannheim).

Cloning of the cDNA-introduction

We have purified this activity to almost homogeneity and demonstrated that it migrates ~50 kDa (Fig. 2). We have thus established that an enzyme that *bona fide* demethylates DNA exists, we have established a protocol to purify the enzyme, assays to determine

the activity and established that it has high affinity to methylated methyl-CpG containing DNA. It is clear that to fully utilize the potential of DNA demethylase as a diagnostic, therapeutic and biotechnology tool in transgenic cells, animal and plants, to develop high throughput assay for inhibitors and activators of the enzyme, as well as to understand its mechanism of action, one needs to obtain the cDNA sequence.

The following principles have been used in cloning of the cDNA for demethylase:

First, the demethylase has high affinity to methylated DNA. Second, the demethylase transforms methylated cytosine in DNA to cytosine. Third, the demethylase releases the methyl group as a volatile compound. Fourth, the demethylase cDNA encodes a protein of an apparent size of 45-66 kDa.

Identification of a lead candidate by homology search of dbEST

Previous reports have shown that proteins interacting with methylated DNA share a common domain (MBD) (Cross, S. H. et al. (1997) *Nature Genet.* **16**:256-259). Assuming that demethylase interacts with methylated DNA, it was predicted that it will bear a methylated DNA binding domain (MBD). The dbEST database was searched using TBLASTN (NCBI) search engine and identified a novel expression tag cDNA (from a T-cell lymphoma *Homo sapiens* cDNA 5'end) and the mouse homologue (gb/W97165/W97165 mf90g05.r1 from Soares mouse embryo NbME13.5) with unknown function that bears homology to the MBD (Fig. 3) (gb/AA361957/AA361957 EST71295). The genbank database was searched to verify that it is a novel cDNA that has not been included in genbank.

Amplification of a 201 bp cDNA fragment bearing the novel cDNA sequence

To generate a probe for screening a library for the putative demethylase cDNA we reverse transcribed and amplified a 201 bp fragment bearing the sequence identified in the search from human lung cancer cell line A549. The following primers were used for amplification: sense 5'CTGGCAAGAGCGATGTC 3', antisense 5'AGTCTGGTTTACCCTTATTTTG 3'. Putative candidate fragments of the predicted size were subcloned into the PCR cloning vector pCR2.1 and the identity of the sequence was determined by dideoxy chain termination nucleotide sequencing.

Cloning of demethylase cDNA from a cDNA library prepared from HeLa cells RNA

A cDNA library from HeLa cells was screened with the subcloned ^{32}P-labeled cDNA probe described above and following 4 rounds of serial dilutions cloning a number of candidate cDNAs were identified. The largest insert was of 1.5 kb size and its sequence identity with the cDNA bearing the MBD was determined by sequencing. The cDNA is novel and has no homologue in genbank and no function has ever been assigned to it (Fig. 4).

Northern blot analysis with the putative demethylase cDNA

To verify that the identified cDNA encodes an mRNA expressed in human cells and that it bears almost the entire mRNA sequence that it encodes, we hybridized a Northern blot bearing RNA from different human tissues with the ^{32}P-labeled putative demethylase cDNA. The probe identified a single prominent band of 1.5 kb

mRNA in all human tissues suggesting that the cloned cDNA does not represent a highly repetitive RNA but rather a mRNA encoded by a single or low copy number gene (Fig. 5).

In vitro translation of the putative demethylase cDNA

To verify that the cloned cDNA encodes a functional protein and to test whether it encodes a functional demethylase, we subcloned it into a pcDNA3.1/His Xpress (Invitrogen) expression vector in all three putative translation frames (A, B &C). The plasmid was *in vitro* transcribed and translated using Promega TNT system in the presence of ^{35}S-methionine and the resulting translation products were resolved by PAGE electrophoresis and autoradiography (Fig. 6). As observed in Fig. 6 a translation product of the predicted 45-66 kDa size was initiated from the pcDNA/HisAdemethylase construct demonstrating that it is in frame with the 6xHistidine tag included in the construct. The histidine tag allows for purification of the *in vitro* translated product using the ProBond™ nickel column. The putative demethylase cDNA was inserted in the antisense orientation into the pcDNA3.1/His Xpress vector (Fig. 7). The sense expression vector allows for expression of demethylase in vertebrate organisms to alter methylation patterns, to differentiate cells, to generate stem cells for therapeutics and to enhance expression of foreign viruses and genes by preventing their methylation. The antisense expression vector allows inhibition of demethylase activity in cancer cells and inhibiting tumorigenesis. Inhibition of demethylase by inhibitors

of demethylase *in vitro* inhibits tumorigenesis (Fig. 8).

In vitro translated demethylase releases volatile methyl groups from methylated DNA

To demonstrate that the cloned cDNA expresses functional DNA demethylase activity, we incubated 10 ng of [^{3}H]-CH_3-DNA with either 15 µl of buffer L, 15 µl of Probond™ purified *in vitro* translated demethylase cDNA or 20 µl of DEAE-Sephadex™ fraction of demethylase purified from A549 human lung cancer cells. The tube was placed in a sealed scintillation vial containing scintillation cocktail and volatilization was determined by measuring the transfer of [^{3}H]-CH_3 from the aqueous phase in the reaction tube to the scintillation cocktail surrounding the tube in a scintillation counter. To verify that the volatilised counts are *bona fide* ^{3}H counts a spectrum analysis was performed. As observed in Fig. 9, both demethylase prepared from A549 cells as well as *in vitro* translated demethylase catalyze the volatilization of ^{3}H-CH_3 from DNA and its transfer into the scintillation cocktail.

In vitro translated demethylase cDNA transforms CH_3-cytosine in DNA to cytosine in DNA

To determine whether the cloned cDNA can catalyze the removal of methyl groups from cytosine, we incubated either *in vitro* translated demethylase, DEAE purified demethylase from A549 cells or buffer L with a methyl-dC^{32}pdG]n double stranded oligomer for eight hours. The reaction products were digested to 3' mononucleotides and analyzed on TLC and the position of methylated and nonmethylated cytosines was determined. Control nonmethylated (NM) [dC^{32}pdG]n substrate was

digested to 3' mononucleotides and loaded on the TLC plate to indicate the expected position of dCMP. As seen in Fig. 10, the *in vitro* translated demethylase cDNA catalyzes the transformation of methylated cytosines to cytosines in DNA since 60% of methylated cytosines has been transformed to cytosines by *in vitro* translated demethylase.

In vitro translated demethylase alters the methylation pattern of DNA using an _in vitro_ methylated SK plasmid as an example

Plasmid pBluescript SK was methylated with M. Sss1 at all CCGG sequences and incubated with either buffer L, demethylase purified from cancer cells and *in vitro* translated demethylase. demethylase treated (methylated SK+dMTase) and nontreated plasmid (methylated SK) as well as nonmethylated SK plasmid were digested with EcoRII, DpnI, MspI and HpaII. The digestion products were analyzed on a 2% agarose gel, blotted onto a Hybond™ N+ filter and hybridized with a 32P labeled SK probe. *In vitro* translated demethylase can alter the methylation pattern of the methylated plasmid as indicated by the appearance of HpaII digested fragments following incubation (Fig. 11). The expected HpaII/MspI fragments are indicated on the right side of the Fig. 11.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the

art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A DNA demethylase enzyme having about 50 to about 60 KDa, and wherein said DNA demethylase enzyme is overexpressed in cancer cells.

2. A cDNA encoding human demethylase which comprises a sequence set forth in Fig. 4.

3. A cDNA homologous to the cDNA of claim 2, wherein said cDNA encoding mouse demethylase.

4. The use of the expression of demethylase cDNA of claims 2 or 3 to alter DNA methylation patterns of DNA *in vitro* in cells or *in vivo* in animals and in plants.

5. The use of claim 4, wherein said demethylase cDNA expression is under the direction of mammalian promoters.

6. The use of claim 5, wherein said promoter is CMV.

7. The use of claim 4, wherein said demethylase cDNA expression is under plant specific promoters to alter methylation in plants and to allow for altering states of development of plants and expression of foreign genes in plants.

8. The use of claim 4, wherein said demethylase cDNA expression is in the antisense orientation to inhibit demethylase in cancer cells for therapeutic processes.

9. The use of claim 9, wherein expression of demethylase cDNA in mammalian cells is to alter their differentiation state and to generate stem cells for therapeutics, cells for animal cloning and to improve expression of foreign genes.

10. The use of the expression of demethylase cDNA of claims 2 or 3 in bacterial or insect cells for production of large amounts of demethylase.

11. The use of the expression of demethylase cDNA of claims 2 or 3 for the production of protein in vertebrate, insect or bacterial cells.

12. The use of claim 11 for producing antibodies against demethylase.

13. The use of the sequence of demethylase cDNA of claim 2 as a template to design antisense oligonucleotides and ribozymes.

14. The use of the predicted peptide sequence of demethylase cDNA of claim 2 to produce polyclonal or monoclonal antibodies against demethylase.

15. The use of expression of cDNA of claim 2 or 3 in two hybrid systems in yeast to identify proteins interacting with demethylase for diagnostic and therapeutic purposes.

16. The use of expression of cDNA of claim 2 or 3 in bacterial, vertebrate or insect cells to produce large amounts of demethylase for high throughput screening of demethylase inhibitors for therapeutics and biotechnology.

17. A volatile assay for high throughput screening of demethylase inhibitors as therapeutics and anticancer agents which comprises the steps of:

a) using transcribed and translated demethylase cDNA of claim 2 or 3 *in vitro* to convert methyl-cytosine present in methylated DNA samples to cytosine present in DNA and volatilise methyl group;

b) determining the absence or minute amount of volatilise methyl group as an indication of an active demethylase inhibitor.

18. A volatile assay for the diagnostics of cancer in a patient sample which comprises the steps of:

a) using transcribed and translated demethylase cDNA of claim 2 or 3 *in vitro* to convert methyl-cytosine present in methylated DNA patient sample to cytosine present in DNA and volatilise methyl group;

b) determining the presence or minute amount of volatilise methyl group as an indication of cancer in said patient sample.

19. Use of an antagonist or inhibitor of DNA demethylase of claim 1 or 2 for the manufacture of a medicament for cancer treatment, for restoring an aberrant methylation pattern in a patient DNA, or for changing a methylation pattern in a patient DNA.

20. Use according to claim 19, wherein said antagonist is a double stranded oligonucleotide that inhibits demethylase at a Ki of 50nM.

21. Use according to claim 20, wherein said oligonucleotide is $(C^mGC^mGC^mGC^mG)$.
$(G^mCG^mCG^mCG^mC)_n$

22. Use according to claim 19, wherein the inhibitor comprises an anti-DNA demethylase antibody or an antisense of DNA demethylase.

23. Use according to one of claims 19 or 22, wherein the change of the methylation pattern activates a silent gene.

24. Use according to claim 23, wherein the activation of a silent gene permits the correction of genetic defect.

25. Use according to claim 24, wherein said genetic defect is β-thalassemia or sickle cell anemia.

26. Use of the demethylase of claim 1, for removing methyl groups on DNA *in vitro*.

27. Use of the demethylase of claim 1 or its cDNA of claim 2, for changing the state of differentiation of a cell to allow gene therapy, stem cell selection or cell cloning.

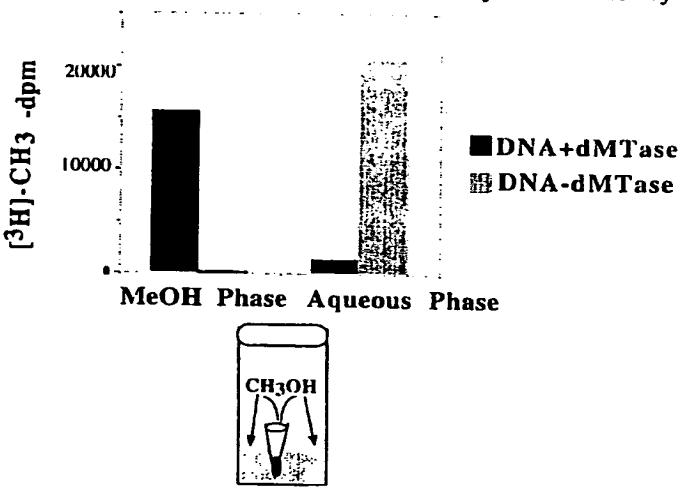
28. Use of the demethylase of claim 1 or its cDNA, of claim 2 for inhibiting methylation in cancer cells using vector mediated gene therapy.

29. An assay for the diagnostic of cancer in a patient, which comprises determining the level of expression of DNA demethylase of claim 1 in a sample

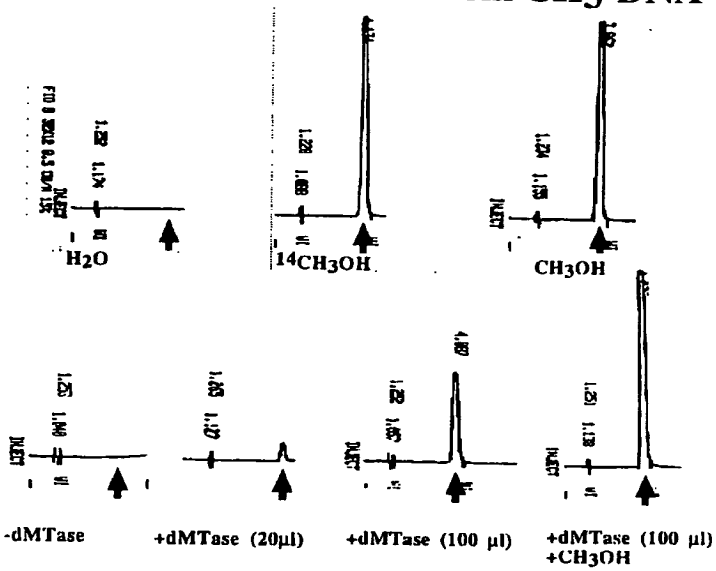
from said patient, wherein overexpression of said DNA demethylase is indicative of cancer cells.

Fig. 1

Volatilization of $[^3H]$-CH_3 from methylated DNA by dMTase



dMTase releases methanol from CH_3-DNA



GC analysis of volatile methanol released from demethylase assay

Figure 2: Volatile and CpG assay of Fraction 63 purified using gel eletrophoresis

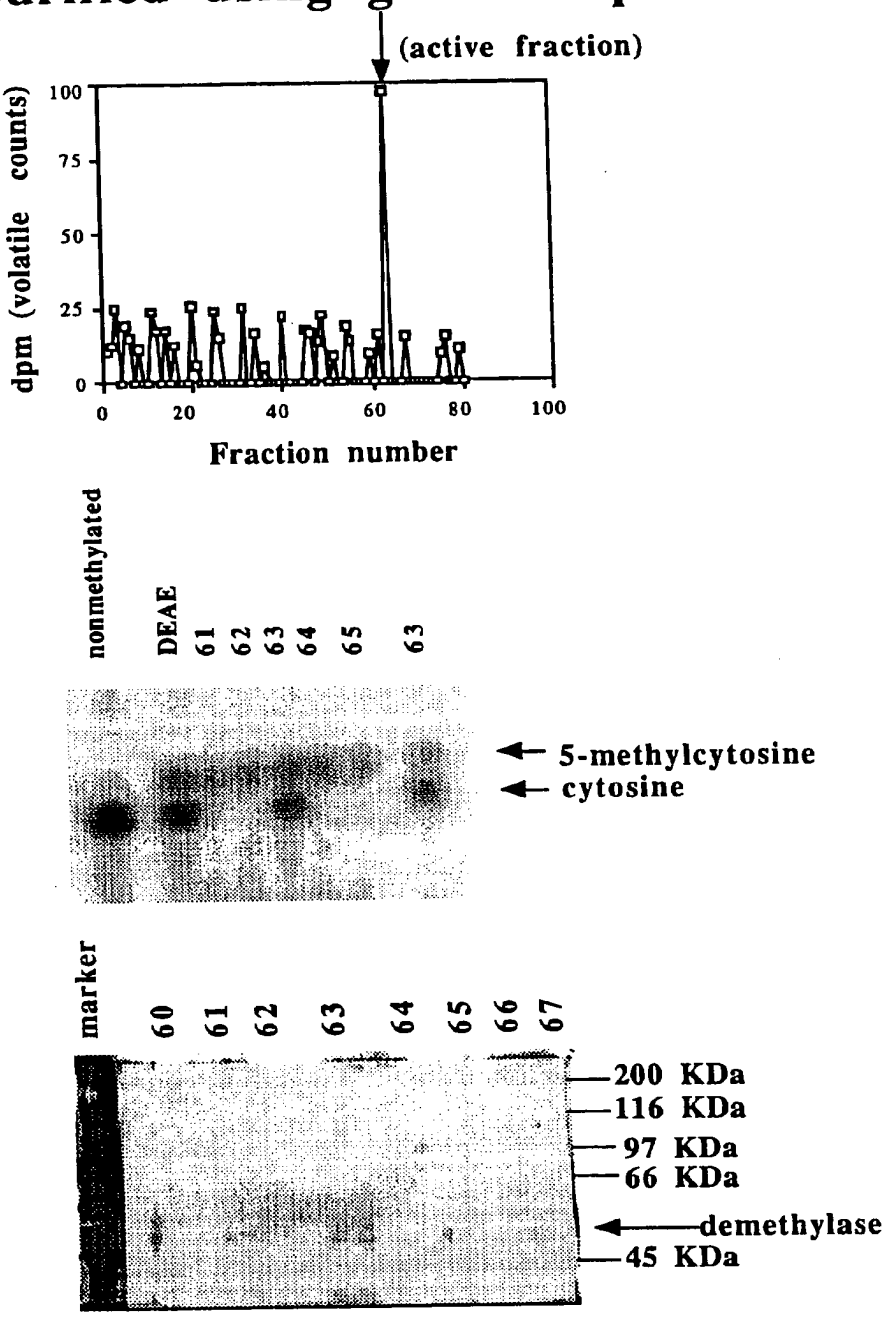


Fig. 3:

Alignment between the MDB domain of MECP-1 and demethylase

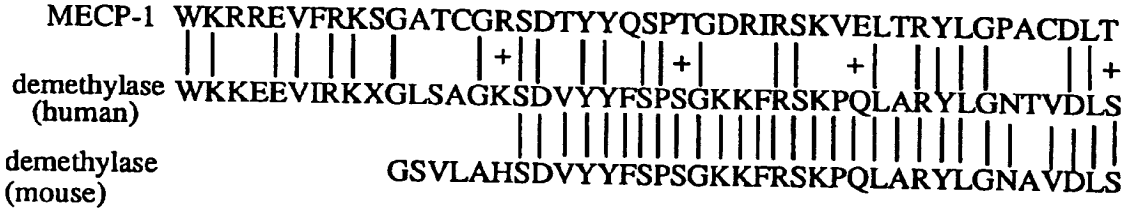


Figure 4:

cDNA sequence of the human Demethylase (1364)

GGAGCCGGTCCCTTTCCCGTCGGGGAGCGCGGGGCGGGGCCAGGGGACCCGGGCCACGGAGAGCGGGAAGAGGATG
GATTGCCCGGCCCTCCCCCCCGGATGGAAGAAGGAGGAAGTGATCCGAAAATCTGGGCTAAGTGCTGGCAAGAGCGATG
TCTACTACTTCAGTCCAAGTGGTAAGAAGTTCAGAAGCAAGCCTCAGTTGGCAAGGTACCTGGGAAATACTGTTGATCTCA
GCAGTTTTGACTTCAGAACTGGAAAGATGATGCCTAGTAAATTACAGAAGAACAAACAGAGACTGCGAAACGATCCTCTCA
ATCAAAATAAGGGTAAACCAGACTTGAATACAACATTGCCAATTAGACAAACAGCATCAATTTTCAAACAACCGGTAACCA
AAGTCACAAATCATCCTAGTAATAAAGTGAAATCAGACCCACAACGAATGAATGAACAGCCACGTCAGCTTTTCTGGGAGA
AGAGGCTACAAGGACTTAGTGCATCAGATGTAACAGAACAAATTATAAAAACCATGGAACTACCCAAAGGTCTTCAAGGA
GTTGGTCCAGGTAGCAATGATGAGACCCTTTTATCTGCTGTTGCCAGTGCTTTGCACACAAGCTCTGCGCCAATCACAGGG
CAAGTCTCCGCTGCTGTGGAAAAGAACCCTGCTGTTTGGCTTAACACATCTCAACCCCTCTGCAAAGCTTTTATTGTCAC
AGATGAAGACATCAGGAAACAGGAAGAGCGAGTACAGCAAGTACGCAAGATATTGGAAGATGCACTGATGGCAGACATC
TTGTCGCGAGCTGCTGATACAGAAGAGATGGATATTGAAATGGACAGTGGAGATGAAGCCTAAGAATATGATCAGGTAAC
TTTCGACCGACTTTCCCCAAGAGAAAATTCCTAGAAATTGAACAAAAATGTTTCCACTGGCTTTTGCCTGTAAGAAAAGAA
ATGTACCCGAGCACATAGAGCTTTTTAATAGCACTAACCAATGCCTTTTTAGATGTATTTTTGATGTATATATCTATTAT
TCAAAAAATCATGTTTATTTTGAGTCCTAGGACTTAAAATTAGTCTTTTGTAATATCAAGCAGGACCCTAAGATGAAGCT
GAGCTTTTGATGCCAGGTGCAATCTACTGGAAATGTAGCACTTACGTAAAACATTTGTTTCCCCCACAGTTTTAATAAGA
ACAGATCAGGAATTCTAAATAAATTTCCCAGTTAAAGATTATTGTGACTTCACTGTATATAAACATATTTTTATACTTTA
TTGAAAGGGGACACCTGTACATTCTTCCATCATCACTGTAAAGACAAATAAATGATTATATTCACAGACAAAAAAAAAAA
AAA

Open Reading Frame and Translation to Amino Acid Sequence

Open reading frame (789 bps)

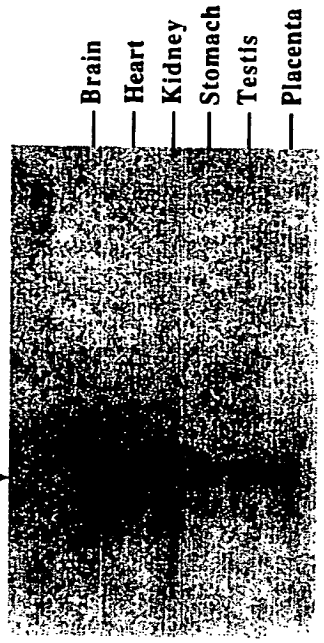
ATGGATTGCCCGGCCCTCCCCCCCGGATGGAAGAAGGAGGAAGTGATCCGAAAATCTGGGCTAAGTGCTGGCAAGAGCG
ATGTCTACTACTTCAGTCCAAGTGGTAAGAAGTTCAGAAGCAAGCCTCAGTTGGCAAGGTACCTGGGAAATACTGTTGATC
TCAGCAGTTTTGACTTCAGAACTGGAAAGATGATGCCTAGTAAATTACAGAAGAACAAACAGAGACTGCGAAACGATCCT
CTCAATCAAAATAAGGGTAAACCAGACTTGAATACAACATTGCCAATTAGACAAACAGCATCAATTTTCAAACAACCGGT
AACCAAAGTCACAAATCATCCTAGTAATAAAGTGAAATCAGACCCACAACGAATGAATGAACAGCCACGTCAGCTTTTCT
GGGAGAAGAGGCTACAAGGACTTAGTGCATCAGATGTAACAGAACAAATTATAAAAACCATGGAACTACCCAAAGGTCTT
CAAGGAGTTGGTCCAGGTAGCAATGATGAGACCCTTTTATCTGCTGTTGCCAGTGCTTTGCACACAAGCTCTGCGCCAAT
CACAGGGCAAGTCTCCGCTGCTGTGGAAAAGAACCCTGCTGTTTGGCTTAACACATCTCAACCCCTCTGCAAAGCTTTTA
TTGTCACAGATGAAGACATCAGGAAACAGGAAGAGCGAGTACAGCAAGTACGCAAGATATTGGAAGATGCACTGATGGCA
GACATCTTGTCGCGAGCTGCTGATACAGAAGAGATGGATATTGAAATGGACAGTGGAGATGAAGCCTAA

Translation (262 amino acids)

MDCPALPPGWKKEEVIRKSGLSAGKSDVYYFSPSGKKFRSKPQLARYLGNTVDLSSFDFRTGKMMPSKLQKNKQRLRNDP
LNQNKGKPDLNTTLPIRQTASIFKQPVTKVTNHPSNKVKSDPQRMNEQPRQLFWEKRLQGLSASDVTEQIIKTMELPKGL
QGVGPGSNDETLLSAVASALHTSSAPTTGQVSAAVEKNPAVWLNTSQPLCKAFIVTDEDIRKQEERVQQVRKILEDALMA
DILSRAADTEEMDIEMDSGDEA.

Figure 5.

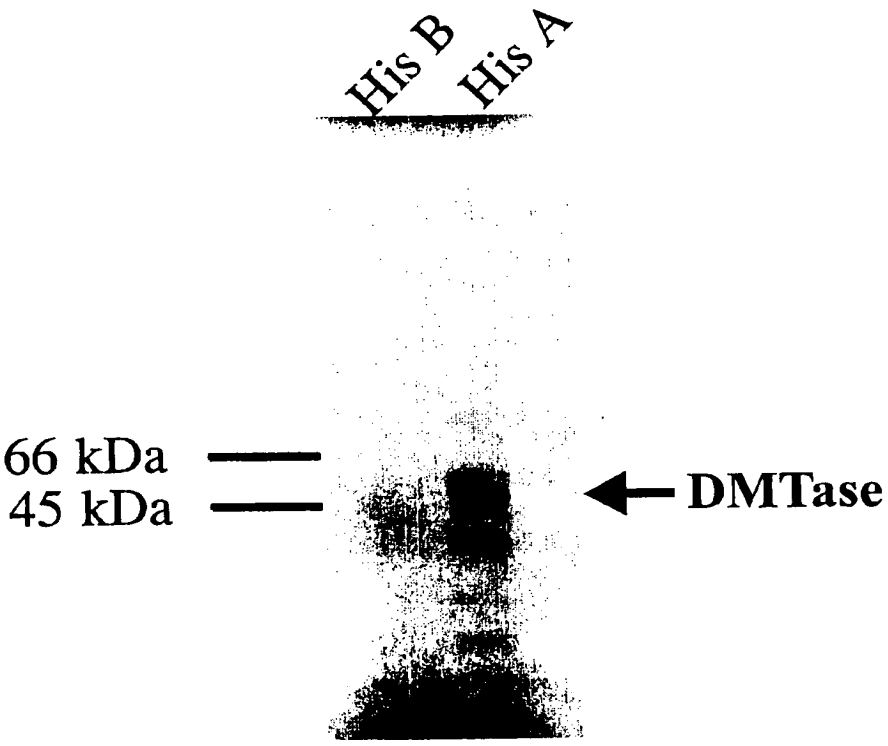
Northern blot analysis of demethylase expression from different human tissues



2 μg Poly A+ mRNA from human tissues (Brain, Heart, Kidney, Stomach, Testis, Placenta: Origene Inc) probed with a ^{32}P α dCTP labeled random primed fragment from the human dMTase cDNA.

Figure 6.

In vitro translated demethylase



The dMTase cDNA was cloned into His tagged mammalian expression vectors, containing a T7 RNA polymerase promoter and were subjected to a T7 rabbit reticulocyte coupled *in vitro* transcription and translation reaction in the presence of (35) S methionine. The product of the reaction migrated with a relative mobility between 66-45 kDa. A frame shift of one base (His B construct) resulted in an early termination

Human dMTase cDNA was cloned from a Hela cell cDNA λ triplex library and cre-lux excised to result in pTriplex containing dMTase. A Not-I fragment containing the entire cDNA clone was sub cloned into pRetrotet-On in both sense and antisense orientations for use as an inducible (with tetracyclin) expresser (sense) or inhibitor (antisense) with of dMTase. dMTase cDNA was also cloned into pcDNA 3.1/His A in both orientations. The CMV promoter of this vector along with the His-Express tags will allow for robust expression and subsequent tracking of dMTase.

Figure 7

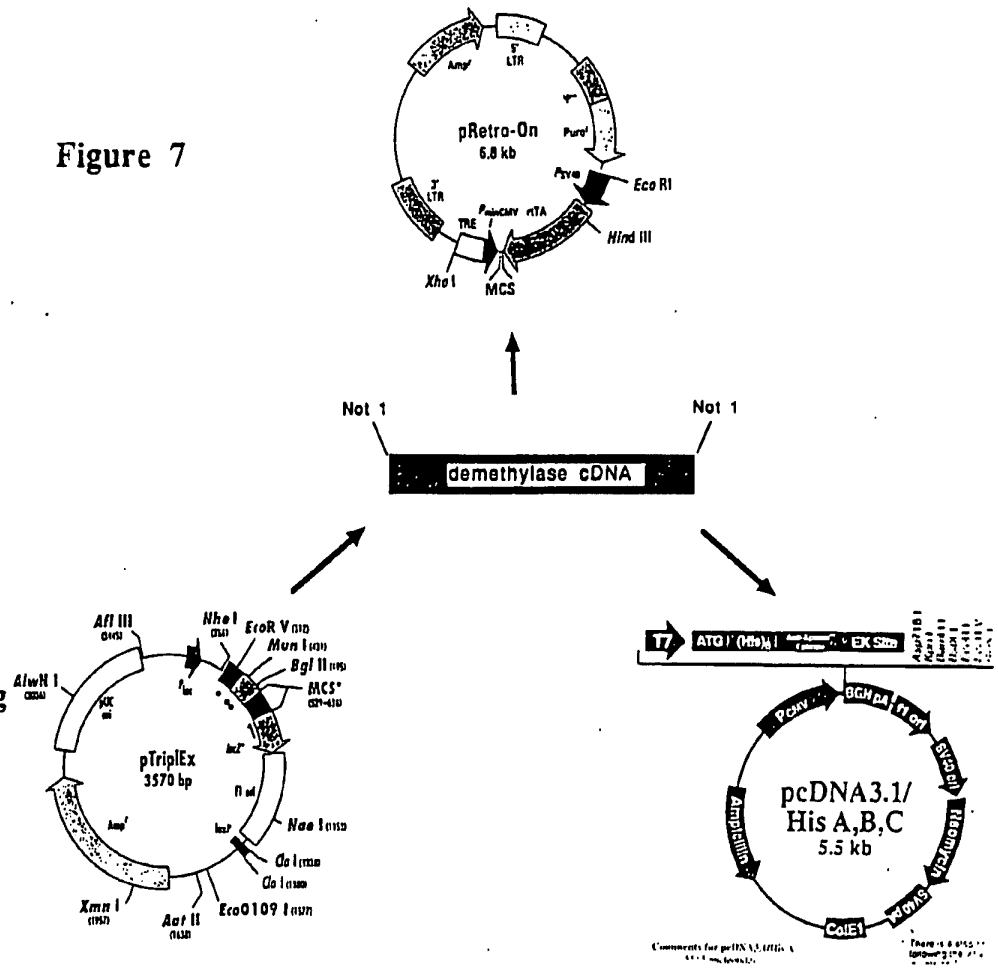
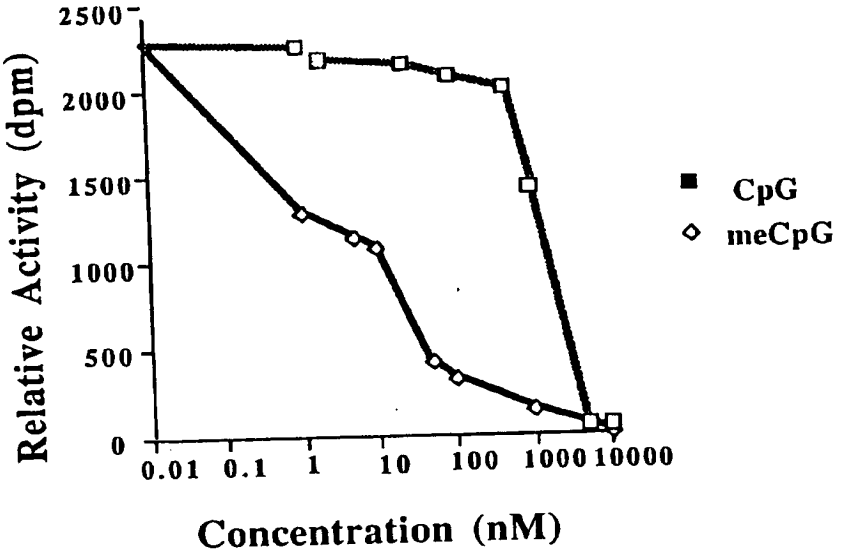


Figure 8

Inhibition of dMTase in vitro by a specific inhibitor



Oligo[methyldCpG]6 is a potent inhibitor of demethylase as determined by an in vitro demethylase assay oligo[dCpdG] is a control.

Inhibition of tumorigenesis in vitro by an inhibitor of dMTase

Human lung carcinoma cells A549 were treated with different concentrations of dMTase inhibitor for three treatments and were then plated onto soft agar for determining tumorigenesis in vitro. Cells treated with dMTase inhibitor are severely inhibited in their ability to form colonies on soft agar.

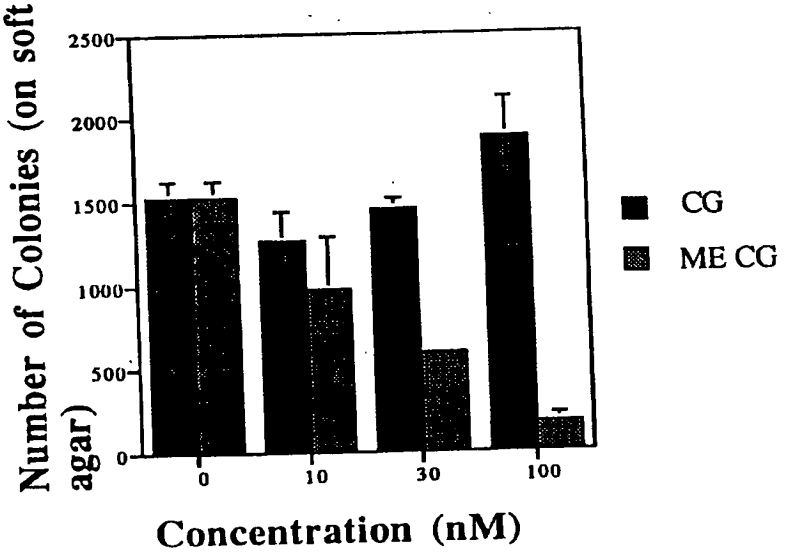


Figure 9: Removal and volatilization of [3H]CH_3 from methyl CpG DNA catalyzed by in vitro translated demethylase

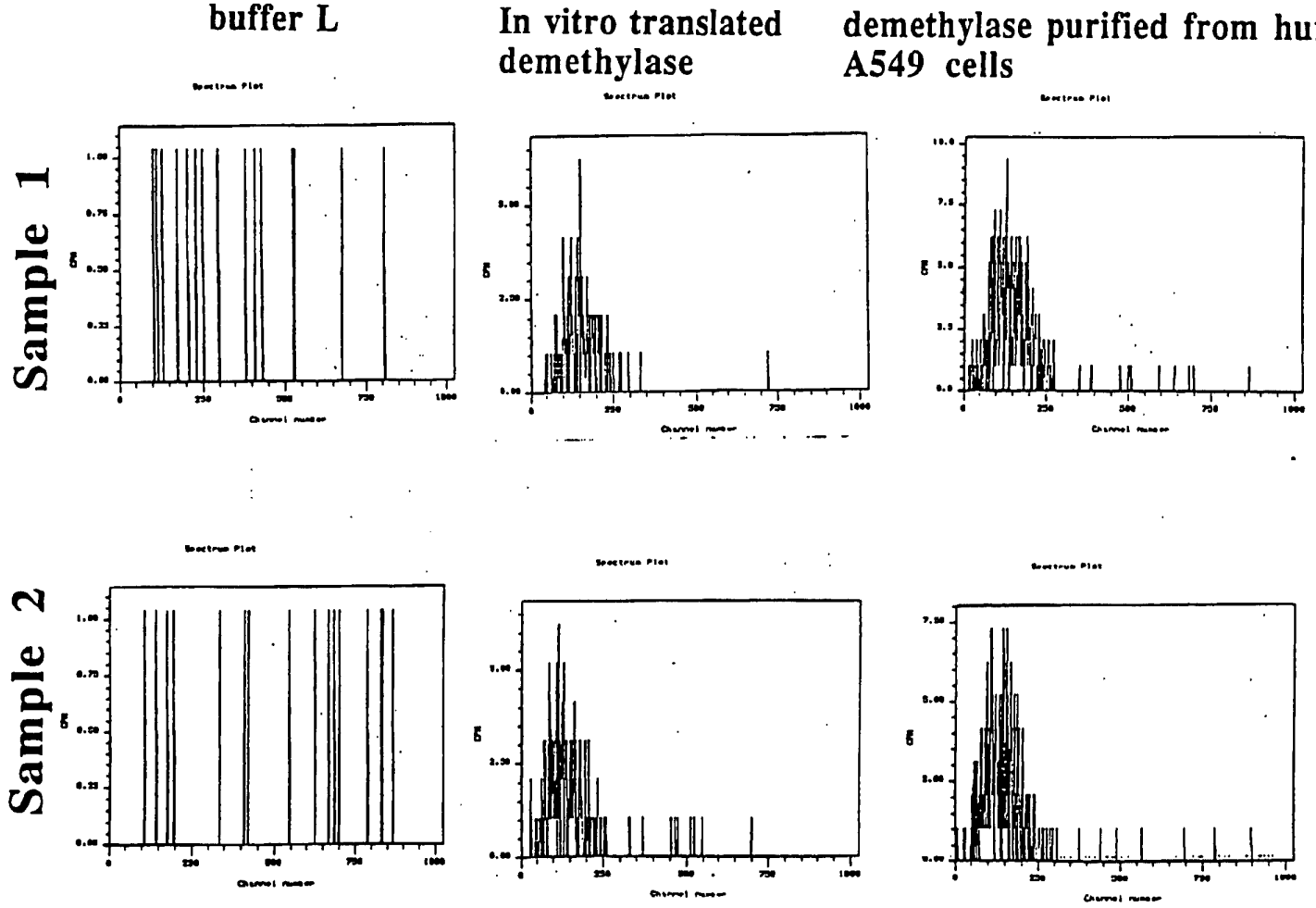


Figure 10: Demethylation of methylated CpG DNA by invitro translated demethylase and DEAE purified demethylase

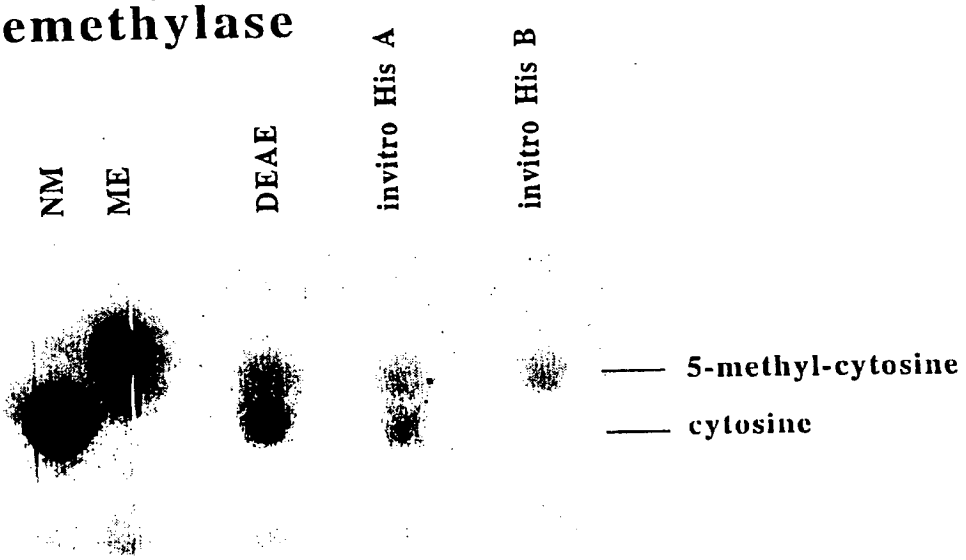


Figure 11: demethylation of Plasmid pBluescript SK II by invitro translated demethylase